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DNA binding site of the growth factor-inducible protein Zif268

("zinc fingers"/transcription factor/cell growth)

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ABSTRACT **Zif268, a zinc finger protein whose mRNA is rapidly activated in cells exposed to growth factors or other signaling agents, is thought to play a role in regulating the genetic program induced by extracellular ligands. We report that Zif268 has one of the characteristics of a transcriptional regulator, namely, sequence-specific binding to DNA. Zif268 synthesized in *Escherichia coli* bound to two sites upstream of the *zif268* gene and to sites in the promoter regions of other genes. The nucleotide sequences responsible for binding were defined by DNase I footprinting, by methylation interference experiments, and by use of synthetic oligonucleotides. From these results we derived the following consensus sequence for a Zif268 high-affinity binding site: GCG(G/T)GGGCG.**

Growth factors and other extracellular ligands induce programs of sequential gene expression that appear to mediate cellular responses to extracellular signals (1–7). Among the genes activated in the first phase of transcription are several encoding known or probable transcription factors that are thought to regulate the genetic program (8–19). One of these presumed transcription factor genes, *zif268* (also known as *NGF-IA, egr-1*, and *Krox-24*), is rapidly induced in various types of cultured cells by growth factors or depolarizing agents and is expressed in a number of tissues and organs of the mouse (12–15), its mRNA being especially abundant in certain regions of the brain (12, 14, 20). The protein encoded by the *zif268* gene contains three tandemly repeated consensus zinc finger sequences, a feature found in the DNA-binding domains of a number of proteins that regulate transcription (21).

To explore the possible role of Zif268 in the regulation of gene activity, it is necessary to identify possible target genes whose expression may be modulated by the protein. We therefore set out to determine whether Zif268 is a sequence-specific DNA-binding protein, as suggested by its zinc finger sequence, and to identify the DNA site to which it binds. As reported in this communication Zif268 binds to specific G+C-rich sequences found in the promoter regions of a number of genes, including genes induced by growth factors.

MATERIALS AND METHODS

Preparation of Zif268. Zif268 *in vitro* translation product was prepared essentially as described (12). The protein produced should contain amino acids 29–533 (12). This *in vitro* translation product was used directly for DNA binding studies. For expression of Zif268 in *Escherichia coli*, an *Nco* I–*Bgl* II fragment of the *zif268* cDNA [nucleotides 338–1994 (12)] was inserted into the T7 promoter-containing vector Pet 8c (22), which had been digested with *Nco* I and *Bam*HI. This resulted in a fusion of the ATG from Pet 8c and the fourth in-frame ATG in the long open reading frame of *zif268* cDNA (12), expected to encode a Zif268 protein of 505 amino acids. The recombinant plasmid was transfected into *E. coli* strain BL21(DE3) and the transfected cells were grown as described (22) to an optical density of about 0.6 before induction of T7 RNA polymerase with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). Bacterial extracts used for DNA binding were prepared as described (23). After solubilization of proteins with 4 M urea the preparation was dialyzed against 20 mM Tris, pH 7.7/50 mM KCl/10 mM $MgCl_2$/1 mM EDTA/10 μM $ZnSO_4$/20% (vol/vol) glycerol/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/1 mM sodium metabisulfite (23). Zif268 in the solubilized extract was about 10% of the total protein.

Gel Retardation Assay for DNA Binding. DNA fragments or double-stranded oligodeoxynucleotides corresponding to the Zif268 binding site were labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase I, as described (24). Binding was carried out in 25 μl of 10 mM Tris, pH 7.5/50 mM NaCl/1 mM dithiothreitol/1 mM EDTA/10 μM $ZnCl_2$/5% glycerol with sonicated salmon sperm DNA at 15–50 μg/ml. *In vitro* translation product or *E. coli* extract containing the Zif268 protein was incubated with all reaction components except labeled DNA for 10 min at room temperature; ^{32}P-labeled DNA was then added to a final concentration of 0.2–0.5 nM, and the mixtures were incubated 20 min longer at room temperature. Competitors used were double-stranded unlabeled oligonucleotides corresponding to the high-affinity *zif268* site, a high-affinity Sp1 site (25), or to *zif268* site mutants; they were included in the preincubation step (before addition of labeled fragment). Following incubation, electrophoresis was carried out as described (24). To quantitate binding, dried gel segments were cut out for liquid scintillation counting.

DNase I Protection Assay. The DNase I footprinting procedure was essentially as described by Graves *et al.* (26). DNA fragments used in footprinting assays were 5′ end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase (New England Biolabs), or in some cases 3′ end-labeled with cordycepin 5′-[α-^{32}P]triphosphate by using terminal deoxynucleotidyltransferase (New England Nuclear). Binding reactions were carried out in 20 mM Hepes, pH 7.4/5 mM dithiothreitol/1 mM $MgCl_2$/60 mM KCl/10 μM $ZnCl_2$. Poly(dI-dC) was used as the nonspecific competitor. Following DNase I cleavage, reaction products were resolved in 6 or 8% polyacrylamide gels containing 8 M urea.

Methylation Interference Assay. A double-stranded oligonucleotide with *Bam*HI and *Bgl* II ends corresponding to positions −616 to −590 of the *zif268* promoter was inserted into the *Bam*HI site of the vector pBLcat-2, which contains the chloramphenicol acetyltransferase coding sequences under the control of the herpes simplex virus thymidine kinase gene (*tk*) promoter (27). *Sph* I–*Xho* I or *Xba* I–*Pst* I fragments containing the inserted oligonucleotide and the majority of the *tk* promoter region were purified and labeled with [α-

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; *tk*, thymidine kinase gene.

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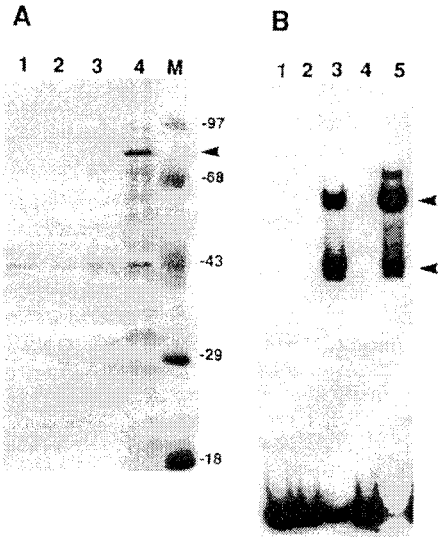

FIG. 1. (*A*) Expression of Zif268 in *E. coli*. BL21(DE3) cells transformed with the Pet 8c vector (lanes 1 and 3) or with a plasmid containing most of the coding region of the *zif268* cDNA (lanes 2 and 4) were grown as described in *Materials and Methods*. The equivalent of 60 μl of culture was analyzed in an SDS/10% polyacrylamide gel. Samples were taken before induction (lanes 1 and 2) or 3 hr after induction of the T7 polymerase with 0.4 mM IPTG (lanes 3 and 4). After electrophoresis, the gel was fixed and stained with Coomassie brilliant blue R. Numbers at right refer to the molecular weights ($M_r \times 10^{-3}$) of the prestained molecular weight standards (lane M). Arrowhead points to the major induced product of the Zif268 expression construct. Another, fainter induced band is also present, which may represent a degradation product of Zif268. (*B*) DNA binding activity of Zif268 produced in *E. coli*, analyzed by gel retardation. Bacterial extract was prepared from cells harboring the Pet 8c/Zif268 plasmid after 3 hr of induction with 0.4 mM IPTG. The probe, a 182-base-pair *Apa* I–*Xma* I fragment (nucleotides −735 to −554) of the *zif268* promoter labeled at the *Xma* I end, was present at a final concentration of 1 nM in the binding reaction mixture. Lane 1, probe alone, no protein; lanes 2 and 4, probe incubated with 1.5 μg (lane 2) or 3 μg (lane 4) of Pet 8c control extract. Lanes 3 and 5, probe incubated with 1.5 μg (lane 3) or 3 μg (lane 5) of Zif268 extract. Arrowheads point to two major complexes formed.

^{32}P]dCTP and [α-^{32}P]dATP at the *Xho* I or *Xba* I ends by using the Klenow fragment of DNA polymerase I. Methylation interference was carried out essentially as described (28). Conditions for the binding reactions were as described above for the gel retardation assay, except on a larger scale. DNA–protein complexes and free probe were separated electrophoretically, isolated, and analyzed as described (29).

RESULTS

Detection of DNA Binding by Zif268. On the assumption that Zif268 is a specific DNA-binding protein that might regulate transcription of its own gene, we searched for a binding site upstream of the coding sequence of a previously analyzed genomic clone of *zif268* (12, 30). In the initial experiments, the *in vitro* translation product of RNA tran-

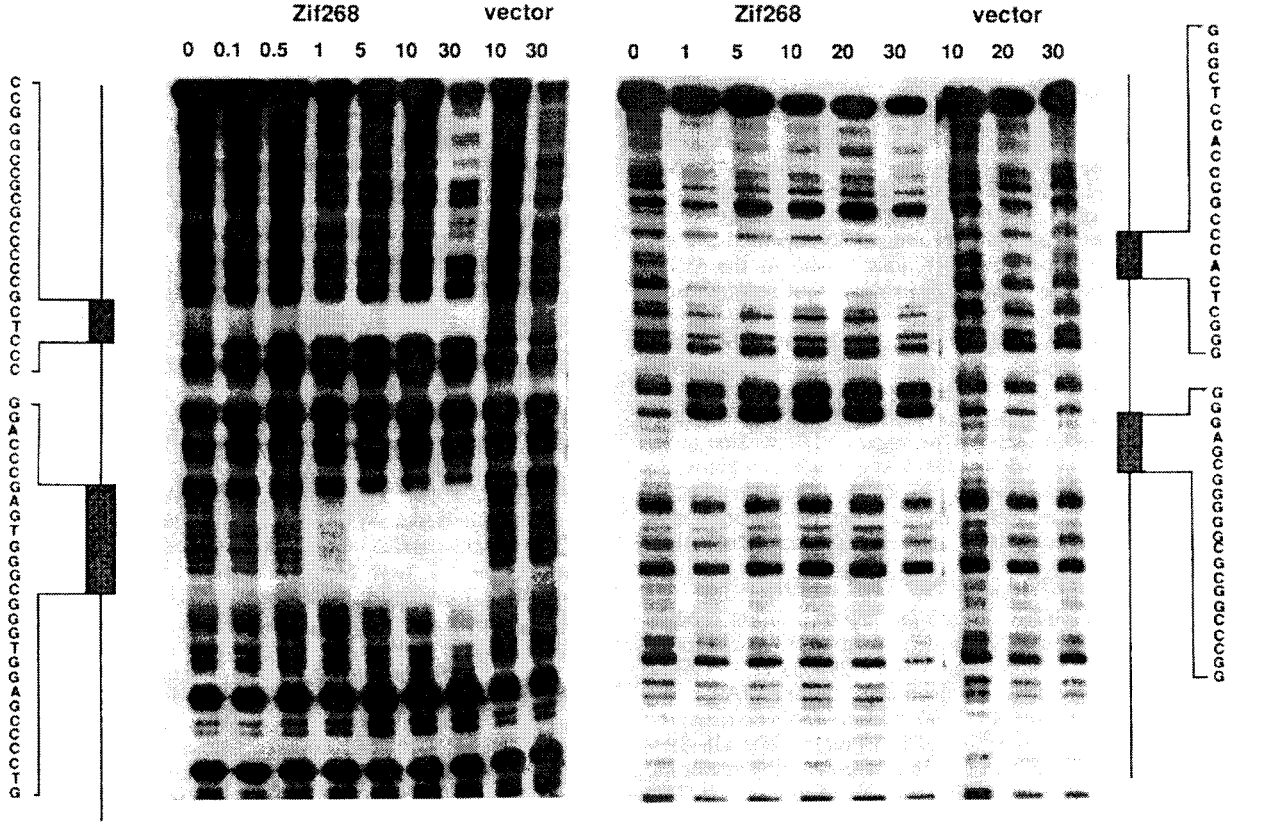

FIG. 2. DNase I protection analysis of *E. coli* Zif268 binding to sites in the *zif268* 5′ flanking region. A 352-base-pair *Apa* I–*Bam*HI fragment containing nucleotides −735 to −384 of the *zif268* promoter was labeled at either end as described in the text. This labeled fragment was incubated with various amounts of Zif268 bacterial extract or control extract (from bacteria containing the vector alone). Numbers above the lanes indicate micrograms of bacterial extract protein used in each reaction. Sequences of the protected regions (shown on the sides of the figure) were determined by comparison with chemical sequencing products of the same labeled fragments (not shown). The left side represents the coding strand, while the right side represents the noncoding strand of the probe.

scribed from *zif268* cDNA was incubated with ^{32}P-end-labeled restriction fragments derived from the upstream region, and the formation of a DNA–protein complex was assessed by gel electrophoresis and autoradiography. Of the three fragments tested, one showed detectable complex formation with the Zif268 translation product (data not shown). To obtain larger amounts of Zif268 we prepared an *E. coli* strain containing a plasmid encoding inducible phage T7 RNA polymerase and a Zif268 plasmid in which transcription was regulated by the T7 promoter (22). Under inducing conditions, about 10% of the solubilized protein of this strain was Zif268 (Fig. 1). An extract of these cells showed marked binding to the *zif268* DNA fragment that bound the Zif268 *in vitro* translation product (Fig. 1), whereas an extract of *E. coli* with only the plasmid vector had no activity. Binding of the ^{32}P-labeled fragment by Zif268 was inhibited by excess unlabeled active fragment but not by an inactive fragment. Further analysis of the active fragment indicated that it has two distinct binding sites (see below). We conclude that Zif268 is a sequence-specific DNA-binding protein and that there are at least two binding sites within 650 nucleotides upstream of the transcription start site of the *zif268* gene.

Sequences of Binding Sites. To further define the Zif268-binding site, DNase I protection assays were performed. Bacterial Zif268 was incubated with a 350-base-pair segment of DNA, labeled at either end, that contained both putative *zif268* binding sites, and the mixture was incubated briefly with DNase I and analyzed by gel electrophoresis and autoradiography (Fig. 2). Two protected regions of differing affinity for Zif268 were found that included related G+C-rich sequences. That these sequences were part of the binding site was confirmed by methylation interference experiments with one of the sites, pinpointing protein–purine contacts in the binding site (Fig. 3). Zif268 contacted all but one of the guanine residues in both strands of the sequence GCGGGGGCG, suggesting that it binds primarily through major-groove contacts. To define the binding-site sequence further, synthetic oligonucleotides with single base-pair substitutions relative to the *zif268* site were tested for binding to Zif268 by competition with an oligonucleotide containing the sequence GCGGGGGCG (Fig. 4). The results indicate that a G·C → T·A change in either of two G·C pairs that contact Zif268 (based on the methylation interference experiment) has a marked effect on binding, whereas change at the position that does not appear to contact the protein has little or no effect.

The transcription factor Sp1 has three zinc fingers that are related to those of Zif268 (23). Since the consensus core Sp1 binding site (GGGCGG) is also similar to that of Zif268 described above (25), we determined whether Zif268 binds to a known Sp1 site. As shown in Fig. 4, an oligonucleotide containing an Sp1 consensus sequence did not compete with the *zif268* site for binding by Zif268. Furthermore, when binding of Zif268 to a natural Sp1 site in the herpes simplex virus *tk* promoter (31) was tested by methylation interference (see Fig. 3) or by DNase I footprinting (data not shown), there was no indication of binding. Finally, when purified Sp1 produced in *E. coli* (23) was incubated with a DNA fragment containing both an Sp1 site and a Zif268 site, only the Sp1 site was protected from DNase I digestion (data not shown). We conclude that Zif268 and Sp1 recognize different, but related, nucleotide sequences.

Other Zif268 Binding Sites. A number of potential Zif268 binding sites were evident in the 5′ upstream sequences of our collection of immediate early genes induced in murine 3T3 cells by serum growth factors (6). Some of these were tested by DNase I footprinting for their ability to bind Zif268 (data not shown); the sequences of the protected regions are shown in Fig. 5. Gene 475 (ref. 6; M. McLane and D.N., unpublished data) has two binding sites and *jun-D* (ref. 32; M. McLane and D. N., unpublished data) has three binding sites. Within each protected region there is a sequence resembling those found upstream of *zif268*. For each of the DNAs tested by footprinting (*zif268*, gene 475, and *jun-D*) the site preferentially protected at low concentrations of Zif268 contained the sequence GCGGGGGCG. Combining these results with the effect of single base-pair substitutions and the results of the methylation protection experiment, we derive the consensus high-affinity sequence GCG$\overset{G}{T}$GGGCG.

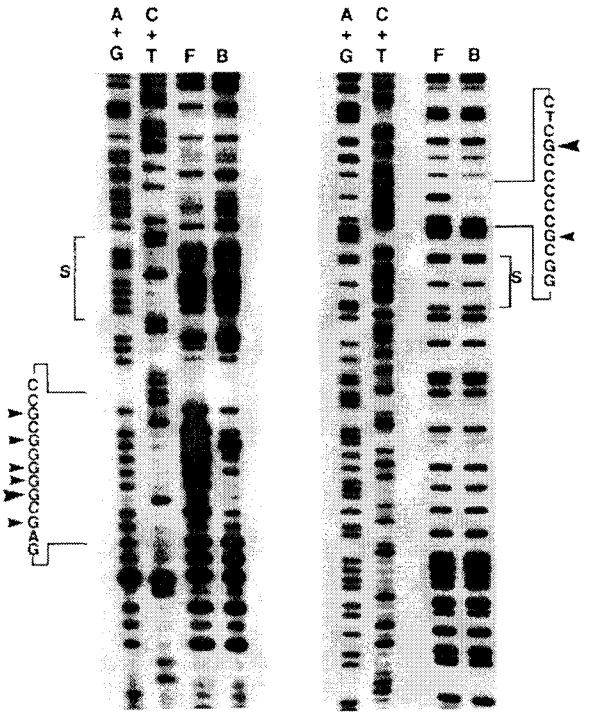

FIG. 3. Methylation interference analysis of *E. coli* Zif268 binding to a cloned *zif268* site. Fragments containing a cloned *zif268* binding site adjacent to the herpes simplex virus *tk* promoter region were labeled at either end. Labeled DNA was treated with dimethyl sulfate, incubated with the Zif268 protein extract, and separated by nondenaturing gel electrophoresis into a protein-bound fraction (lanes B) or unbound fraction (lanes F). Each fraction was cleaved at methylated purine residues and analyzed by electrophoresis in 6% or 8% polyacrylamide/8 M urea sequencing gels. Lanes on the sides of each panel are purine (A+G) and pyrimidine (C+T) sequence tracks of each strand. The sequence of the bound region is indicated on the side of each panel, and the inferred purine contact sites are marked with arrowheads, with the largest ones denoting the most strongly interfering methylated bases. This fragment also contains a known Sp1 site (S) derived from the *tk* promoter sequence (31). Note that cleavage of the Sp1 site is not affected by binding of Zif268.

DISCUSSION

Zif268 is a zinc finger protein whose gene is widely expressed in mouse tissues (12–15). Various growth and differentiation factors and other extracellular agents induce *zif268* expression in cultured cells (6, 13–15, 33). In intact animals, depolarizing stimuli increase *zif268* mRNA levels in the hippocampus (20), and partial hepatectomy induces expression in the liver (34). Recently it was shown that enhanced *zif268* expression in the hippocampus is correlated with the induction of long-term potentiation by localized electrical stimulation (35). Zif268 is therefore likely to be a widespread mediator of cellular responses to a variety of stimuli. Since Zif268 has three contiguous, typical zinc finger sequences, it

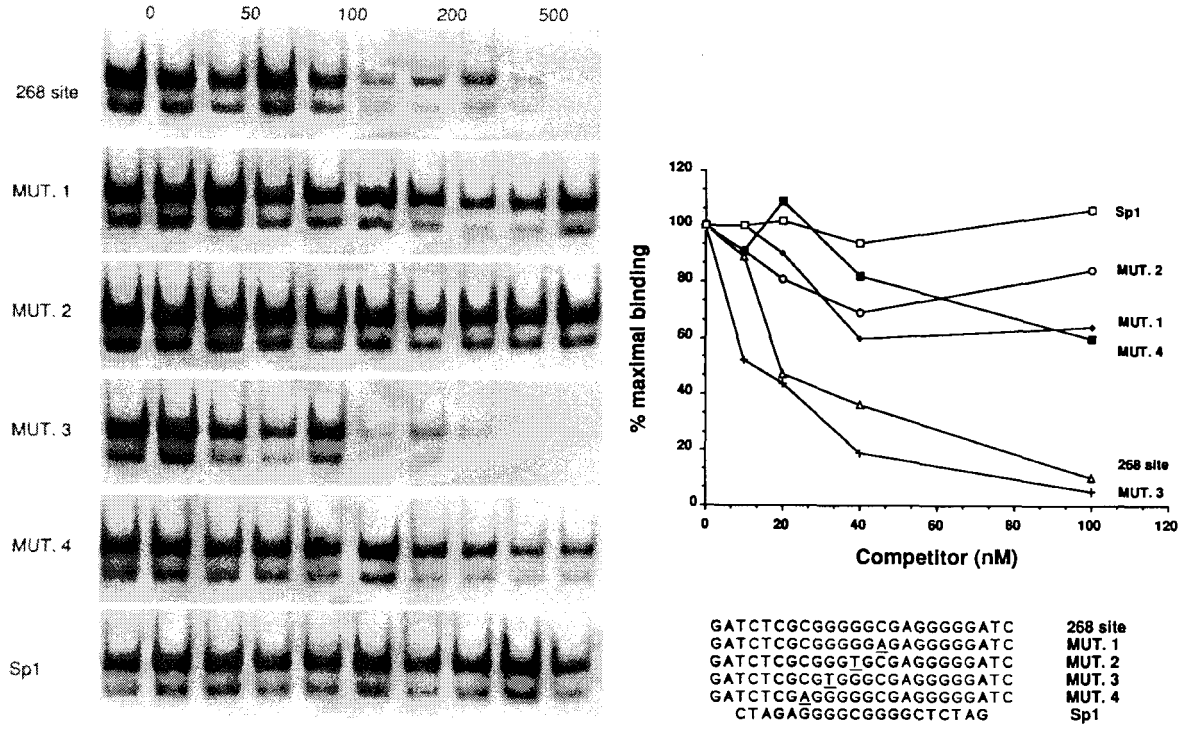

FIG. 4. Gel retardation competition assay. Bacterial Zif268 extract was incubated with 0.2 nM labeled double-stranded oligonucleotide probe corresponding to a *zif268* binding site in the presence of increasing concentrations of various competing double-stranded oligonucleotides. Reaction products were electrophoresed in nondenaturing 6% polyacrylamide gels. (*Left*) Autoradiograms of the gels. Numbers at the top indicate the ratio of competitor to probe for each oligonucleotide (identified at the left). (*Right*) The sequence of each of the competitor DNAs is shown at the bottom, with the mutant sites underlined. Regions of the gels corresponding to the retarded fragments were excised and counted. Percentage of maximal binding is shown as a function of competitor concentration.

has been inferred that Zif268 is a sequence-specific DNA-binding protein that regulates the transcription of other genes; i.e., that it (along with other induced transcription factors) regulates the genetic program induced by extracellular agents. In the present report we have shown that the first part of this inference is correct, by demonstrating that Zif268 binds to a specific sequence in duplex DNA.

The sequence to which Zif268 binds was initially identified by guessing that DNA upstream of the *zif268* gene might have a binding site. In fact there was one strong binding site and one weaker site within the 650 base pairs 5′ to the transcription start site of *zif268*. Once these sequences were identified, a number of sites 5′ to other genes were found by DNase I footprinting. These sequences, together with the binding

CCCGGC	GCGGGGGCG	AGGG	zif268
GTCCCCGAGGTGG	GCGGGTGAG	CCCAGG	zif268
TACGCC	GCGGGGGCG	C	475
C	CTGGGGGCG	TGGCC	475
TGGCGA	GCGGGGGCG	GTGAG	junD
CTCATC	GCGGGGGCC	GAGGCT	junD
GTGC	GCGCGGGCG	GCGCGGGGC	junD
GATCTC	GCGTGGGCG	AGGGGGATC	MUT.3
	GCGG/TGGGCG		CONSENSUS

FIG. 5. Sequences protected by Zif268 binding to DNA fragments from the promoter regions of three immediate early genes. For each gene the first site listed was preferentially protected at low concentrations of Zif268.

properties of synthetic oligonucleotides, were then used to establish a consensus high-affinity binding-site sequence, GCG(G/T)GGGCG. The methylation interference experiment indicates that Zif268 makes extensive major-groove contacts along this sequence.

As previously reported, the three zinc finger sequences of Zif268 are nearly identical to those of Krox-20 (11) and more distantly related to those of Sp1 (23). We therefore anticipate that Krox-20 will bind to a DNA sequence that is very similar, if not identical, to the Zif268 binding site. Since these two proteins are quite different in sequence outside the zinc finger region, the consequences of binding to DNA may be different. In the case of Sp1, the binding site [core consensus sequence GGGCGG (25)] is related to the Zif268 site, but as shown in this report, Zif268 does not show appreciable binding to a typical Sp1 site. It is possible, however, to have partially overlapping Zif268 and Sp1 sites that could bind one or the other protein depending on their relative concentrations. Such competitive binding could have physiological significance.

It is of some interest that one or more Zif268 binding sites or predicted sites are found upstream of a number of immediate early genes induced by growth factors and other extracellular stimuli: *zif268*, gene 475, *nur77* (L. Lau, personal communication), and *jun-B* (G. Schatteman and D.N., unpublished data). The significance of this observation is not known, but one possibility is that Zif268 down-regulates these genes, whose transcription is repressed soon after growth factor-induced activation (6). Other genes also have upstream Zif268 binding-site sequences, including *jun-D*, c-Ha-*ras* (36), *int-2* (37), human c-*abl* (38), rat *hsc73* (39), and genes encoding human histone H3.3 (40), mouse metallothionein (41), the A chain of platelet-derived growth factor (42),

and the mouse neurofilament protein (43). Of particular interest would be the identification of binding sites in promoter regions of genes expressed later in the growth factor-induced genetic program. Such genes are potential targets for activation by Zif268 in the cellular responses mediated by the various ligands that induce Zif268. With the binding site now identified, it will be possible to search for these potential target genes and to determine what effects Zif268 has on their transcription.

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